Ph.D Thesis -Title

"DEVELOPMENT OF VACCINES FOR GOLD FISH HEMATOPOIETIC NECROSIS VIRUS (GFHNV) DISEASE"

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Chapter 1. General Introduction

1.1 Introduction

The growing international trade of ornamental fishes have made its level into multibillion dollar business. About 125 countries are involved in trading more than 2,500 species, of which majority constitutes freshwater fishes (Monticini, 2010; Olivotto et al., 2011; Biondo & Burki, 2020). The total ornamental fish trade from India in 2017 – 18 were recorded as 42 tonnes, that valued about 8.4 crore (US $1.2 million). The common aetiological agents involved in infectious disease in industry comprises of bacteria, viruses, parasites and fungi which accounts about 54,9%, 22,6%, 19.4%, 3,1% respectively. (Kibenge et al., 2012). The mortality due to virus reaches upto 100% and viral infections also predisposes the susceptibility to other secondary diseases.

Goldfishes (*Carassius auratus*) are one among the most traded ornamental fishes worldwide. *C. auratus* is a member of the cyprinid family and like the carp is a native of Eastern Asia. *C. auratus* are affected by goldfish virus type 1 and type 2 (GFV 1, GFV 2) (Shea & Berry, 1984) and also with herpes viruses (Jung & Miyazaki, 1995). About 14 herpes viruses are emphasised in causing fish disease outbreaks. Cyprinid herpes virus effects cyprinids and have made its way into scientific research due to its pathogenicity and the economic losses they cause to the aquaculture industry.

1.1.1 Rationale for selection of Cyprinid herpesvirus-2 (CyHV-2):

India gains about US$1.06 million in the overall ornamental fish trade and its <1% in global market (Rani et al., 2014). The annual growth rate was recorded as 11%.1 in the time period from 1995 to 2014. In India, 90% of export shipping comes from Kolkata, 8% from Mumbai and 2% from Chennai (Ghosh et al., 2003). The mostly shipped ornamental fishes are *Poecilidae, Cyprinidae, Cichlidae, Characidae* and *Anabantidae*. *C. auratus and Cyprinus*

carpio are the frequently traded fish and accounts for a large-scale production. The major challenge is posed by the viral diseases in accounts to the escalating ornamental fish culture sector (Bernoth & Crane, 1995).

Cyprinid herpesvirus-1 (CyHV-1), *cyprinid herpesvirus-2* (CyHV-2), *cyprinid herpesvirus-3* (CyHV-3) are the three resembling herpesviruses known to cause noticeably diverse diseases in cyprinids (Davison et al., 2013). The host-specific herpesviruses have produced much loss in aquaculture globally. *Cyprinid herpesvirus-2* being lethal to *C.auratus* produces about 90% to 100% mortality. The CyHV-2 is also known as herpesviral haematopoietic necrosis virus (HVHNV) or goldfish haematopoietic necrosis virus (GHNV). It is an important pathogen of *C. auratus*, *C. carassius*, *C. gibelio*. This virus is wide spread on *C. auratus* farms and is persistently reported globally. In India, the devastating outcome due to CyHV-2 infection in *C. auratus* farms were first recorded from West Bengal in 2014 (Sahoo et al., 2016). The prevalence rate was 30-100%. It is anticipated that a vaccine and rapid diagnostic tests can be effective for the prevention and control of this virus. In India, vaccination and isolation of CyHV-2 from diseased *C. auratus* has been limited.

Cell lines being one of the methods for detection of virus, we have aimed in developing cell lines from goldfish which will further aid to achieve successful propagation of virus with good viral titres. An important challenge to the accurate detection of CyHV-2 infections is the virus capacity to exist in latent stages. Hence our aim is to develop new PCR primer amplifying major capsid gene for detecting CyHV-2 and to compare the sensitivity of diagnosis with the existing helicase and DNA polymerase genes primers. There was also a need for the field-based diagnosis of CyHV-2 for which we have aimed in developing LAMP and RPA-LFD analysis. As vaccination is an easy, and effective method of protecting fish from diseases, this study also proposes the development of different types of inactivated vaccines and immune gene expression analysis post-vaccination to determine the ideal vaccine candidate to combat the spread of CyHV-2 in goldfish. In this context, following objectives were proposed.

1.1.2 Objectives of the present study

1. To develop a suitable method to propagate the *in vitro* viral titer of CyHV-2 virus using different fish cell lines.
2. To develop sensitive methods for the rapid detection of CyHV-2
3. To develop inactivated vaccines to combat the spread of CyHV-2 in goldfish.

4. To check the potential efficiency of vaccines derived from inactivated cell culture supernatant of CyHV-2

1.2 Review of Literature

1.2.1 *Herpesvirales*

The order *Herpesvirales* holds up the feature of large complex double stranded DNA capable of causing disease. The viruses in the order *Herpesvirales* are commonly termed as herpesviruses (HVs). The HVs ranges from 120 – 260 nm in diameter where it infects and replicate in the eukaryotic nucleus. The order *Herpesvirales* have been classified into 3 separate families based upon their genetic distances among the groups: *Herpesviridae* – viruses affecting reptiles, birds and mammmals, *Alloherpesviridae* – viruses identified in amphibians and fish and *Malacoherpesviridae* – viruses upholding in mollusk (Davison et al., 2009; McGeoch et al., 2006). The *Alloherpesviridae* consists of four genera - *Batrachovirus, Cyprinivirus, Ictalurivirus, and Salmonivirus. The family Alloherpesviridae* comprises of very diverse set of viruses whose genome size varies from 134 kb (*Channel catfish virus*) to 295kb (*Cyprinid herpesvirus-3*) (Lepa & Krzysztof Siwicki, 2012).

1.2.2 *Cyprinivirus*

The International Committee on Taxonomy of Viruses (ICTV) (Pellett et al., 2011) includes four species of viruses in the *Cyprinivirus*, three of them (*Cyprinid herpesvirus 1, Cyprinid herpesvirus 2*, and *Cyprinid herpesvirus 3)* are included in the family *Cyprinidae* in the order *Cypriniformes* and one (*Anguillid herpesvirus 1)* comes under family *Anguillidae* in the order Anguilliformes.

Cyprinid herpesvirus 1 (CyHV-1) also called as carp pox disease, carp herpesvirus and *Herpesvirus cyprini* is one among the earliest detected virus in fish dated almost in 1563 (C. Erickson, 2000) causing epidermal hyperplasia, in common carps. *Cyprinid herpesvirus 2* (CyHV-2), also named as goldfish hematopoietic necrosis virus (GFHNV) mainly causing diseases in *C. auratus*. But the CyHV-2 is also isolated from the Prussian carps, crucian carps and hybrids (Thangaraj et al., 2021). *Cyprinid herpesvirus 3* (CyHV-3) also recognized as Koi herpesvirus (KHV) (Hedrick et al., 2000) and Carp interstitial nephritis and gill necrosis virus (CNGV) (Ronen et al., 2003) causes koi herpesvirus diseases, mainly causing epizootics in *C. carpio* and common carp and it has a genome size of 295kb which is the largest sequenced genome till date (Costes et al., 2008).

1.2.3. *Cyprinid herpesvirus 2* (CyHV-2)

1.2.3.1 Global distribution

CyHV-2 was first detected as the source of epizootics in *C. auratus* in Japan in spring and autumn with a nearly 100% mortality rate (Jung & Miyazaki, 1995). Owing to the global commerce in *C. auratus,* CyHV-2 was further detected in European, Asian, Oceania and South American countries. The worldwide spread of CyHV-2 infections were reported in different countries like Taiwan (Chang et al., 1999), USA (Goodwin et al., 2006a), Australia (Stephens et al., 2004), Hungary (Doszpoly et al., 2011), UK (Ito et al., 2013), Czech Republic (Daněk et al., 2012), China (Jiang et al., 2015; Xiao et al., 2022), Japan (Ito et al., 2013), Italy (Fichi et al., 2013), Switzerland (Giovannini et al., 2016), India (Sahoo et al., 2016)S, France (Boitard et al., 2016), Netherlands (Ito et al., 2017), Germany (Adamek et al., 2018), Turkey (Kalayci et al., 2018) and Poland (Panicz et al., 2019). Even *C. carassius*, *C. gibelio* (Daněk et al., 2012; Xiao et al., 2022) and allogynogenetic *C. carassius* Wu et al. (2013) have been reported recently.

1.2.3.2 Genome

The genomic size of CyHV-2 is 290,455 bp and till date seven complete genome sequencing are done for CyHV-2 in which ST-J1(290,304 bp, NC019495) (Davison et al., 2013) is the isolate from Japan, SY-C1(289,365 bp, KM200722) (Li et al., 2015) an isolate from China, CaHV (275,348 bp, KU199244) isolate from *C. carassius* (Zeng et al., 2016), CyHV2-SY (290,455 bp, KM200722) (Liu et al., 2018), CNDF-TB2015(288,321 bp, MN201961) submitted in 2019 in NCBI, YZ-01 (MK260012, 288,076 bp) isolated in China (Wang, 2021) and SH-01 (290,428 bp, Bankit 2436221) isolated from China (Yang et al., 2022). Based on their isolation loci and the molecular epidemiological surveys pointed out that the strains found in China had close similarity with the C genotype than the J genotype. Later in 2018, Liu et al sequenced a strain CyHV2-SY, where he could find difference from ST- J1 and SY-C1, having deletions or insertions of repeat sequences in ORF10, ORF107, ORF156.

1.2.3.3 Cell lines and virus propagation

Since the start of 1992, attempts have been made to isolate CyHV-2 in vitro. Cell lines used commonly for virological studies gave an unsatisfactory result for the propagation of CyHV-2. The first attempt in using in vitro method for isolation of CyHV-2 was given by Jung & Miyazaki in 1995, where a series of cell lines like fathead minnow (FHM), epithelioma papillosum cyprini (EPC), eel kidney (EK-1), chinook salmon embryo (CHSE-214), rainbow trout gonad (RTG-2) and tilapia ovary (TO-2) were used for CyHV-2 study. The CPE was observed in FHM, EPC and TO-2 cells only. Later on, different cell lines like koi fin (KF-1), bluegill fibroblast 2 (BF-2), goldfish (GF -1), common carp brain (CCB), standard Ryukin Takafumi (SRTF), Ryukin fin (RKF), Cyprinus carpio koi fin (CCKF), Gold fin (GFF), Gibel

Carp Brain (GiCB) were attempted for CyHV-2 isolation. The greatest titre of virus produced by these cells, however, was only $10^{3.0 \pm 0.26}$ - $10^{2.3 \pm 0.30}$ $TCID_{50}$/mL. In 2015, a cell line was developed from Gibel Carp Brain which gave a lead by propagating CyHV-2 upto 50 passages (Ma et al., 2015). The highest viral titer of CyHV-2 was obtained from the GiCB cell line which was about $10^{7.5} \pm 0.37 TCID_{50} ml^{-1}$. A novel cell line, GiCF cell line (*C. auratus gibelio* caudal fin cell line) was established by Lu et al. (2018) which produced a virus titre of $10^{4.9 \pm 0.22}$ $TCID_{50}$/mL which have attained over 30 passages.

1.2.3.4 Vaccine

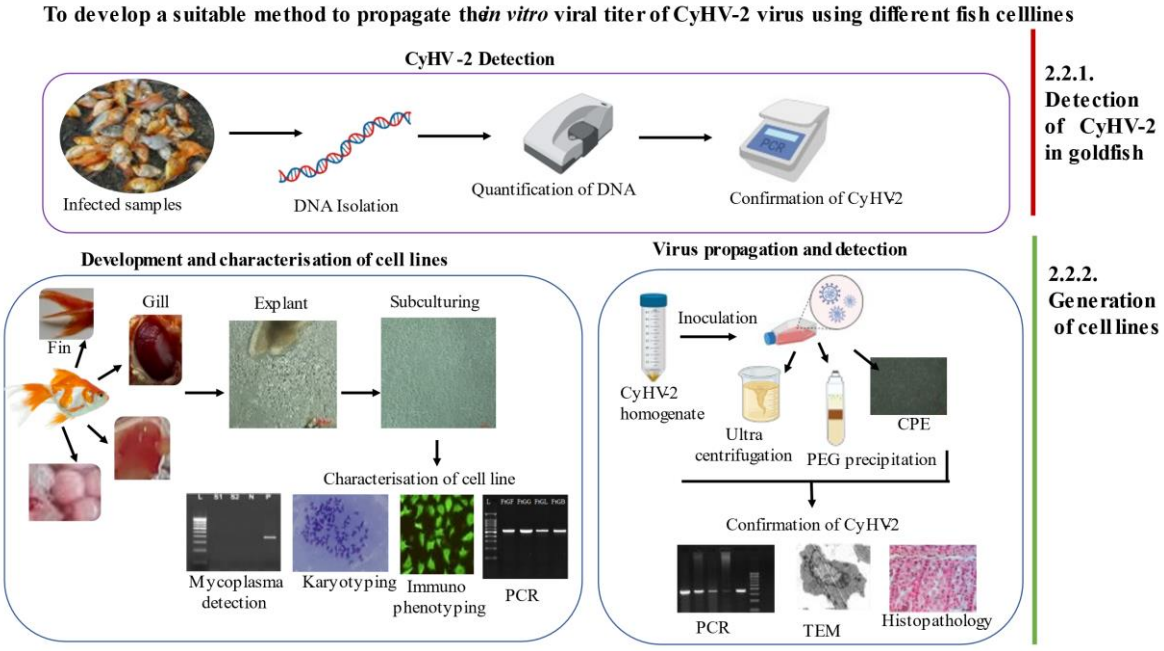
Vaccines are best suitable for preventing viral disease as they develop an immunological memory, thus boosting up the adaptive immune response (McHeyzer-Williams 2005; Sallusto 2009). Different types of vaccines are possible, i.e., inactivated vaccines either adjuvanted or not, live attenuated vaccines, sub-unit vaccines, recombinant vaccines, synthetic vaccines (peptide vaccines) or DNA vaccines (Zhang et al. 2016). Most of the fish vaccines developed are inactivated vaccines as they are safe and can produce strong immune response than killed and DNA vaccines. Recombinant baculovirus (BacCarassius-D4ORFs) were used as live vector vaccine in infected *C. gibelio* which seems to be a potential candidate CyHV-2 vaccine (Li et al., 2019). An adjuvant (β-glucan or anisodamine) was added to the b-propiolactone-inactivated CyHV-2 for improving the efficiency of vaccine and this proved to have a good immunogenic response (Yan et al., 2020).

Chapter 2: To develop a suitable method to propagate the *in vitro* viral titer of CyHV-2 virus using different fish cell lines.

2.1. Introduction

Most of the viruses are host specific and it implies the necessity to produce cell lines of each fish for their virological studies. To the best of our knowledge, until now twenty one adherent cell lines from *C. auratus* had been developed and reported including whole small goldfish, pectoral muscle, and heart muscle (Kroeker, 1964), goldfish fin (CAR) (ATCC, CCL-71), goldfish skin (SJU-1) (Rio et al., 1973), goldfish skin (GFSk-S1) (Lee et al., 1997), goldfish fin GFF (Li & Fukuda, 2003), goldfish muscle (GFM) (Rougée et al., 2007), swim bladder (GFSB) (Rougée et al., 2007), goldfish tail fin (GFTF) (W. Yan et al., 2011), Ryukin Takafumi fin (SRTF) (Ito & Ototake, 2013), Ryukin fin (RKF) (Ito & Ototake, 2013), Ryukin goldfish (RyuF-2) (Shibata et al., 2015), Gibel Carp Brain (GiCB) (Ma et al., 2015), snout (GFSe) (Jing et al., 2016), goldfish kidney (GFKf) (Jing et al., 2016), goldfish heart (GH) (Jing et al., 2017), goldfish brain (GFB) (Xu et al., 2019) and fantail goldfish fin (FtGF) (Dharmaratnam et al., 2020), fantail goldfish brain (FtGB), fantail goldfish liver (FtGL), fantail goldfish gill (FtGG) (Swaminathan et al., 2021), grass goldfish muscle (CAM) (Li et al., 2021). There has been no description of the establishment of cell lines from the target tissue of CyHV-2 including gill and liver and as well brain tissue of the *C. auratus* to propagate CyHV-2 elsewhere except for the GiCB cell line which was found to produce a higher CyHV-2 titer value $10^{7.5\pm0.37}$ $TCID_{50}$/mL of and was passaged over 50 times (Ma et al., 2015).

2.2. Methodology



2.3. Results

Samples of goldfishes were collected from different farms of Kerala, Tamil Nadu and West Bengal. The affected fish showed signs of lethargy, whitish bleached gills and hemorrhages on the body. On post-mortem examination, the fish showed massive necrosis of gills, swollen kidney and spleen, whitish necrotic nodular foci in spleen. Out of all the samples, the fishes collected from three farms only shown positive for CyHV-2 by DNA polymerase PCR. Permissive cell lines for the isolation and propagation of CyHV-2 have been established from various goldfish tissues. Here, we report the development of a cell line, FtGF (Fantail Goldfish Fin), FtGG (Fantail Goldfish Gill), FtGB (Fantail Goldfish Brain) and FtGL (Fantail Goldfish Liver) from caudal fin, gill, brain and liver of goldfish that were tested to attain a good CyHV-2 titre which can be further used for the vaccine development. The growth of the cell lines was standardised at different temperatures and FBS concentrations, where we found that Leibovitz's 15 (L-15) medium containing 20 % FBS incubated at 28 ℃ provided a good growth. Species authentication was confirmed by sequencing the cell lines by 16S rRNA. Karyotyping of the cell lines were done where the chromosome number was diploid (2n = 100). The Population Doubling Time during exponential growth of FtGF, FtGG, FtGL and FtGB cells were determined to be 33.9 h, 41.47 h, 63.43 h and 79.79 h, respectively. The cell lines from fin, gills, liver and brain have been subcultured for 42, 35, 30 and 25 times respectively in L-15 with 10 % FBS. All the four cells showed similar cytopathic effect (CPE) between 3-5 days post infection (dpi) with CyHV-2 and complete destruction of the monolayer was observed at 8-10 dpi. The cell lines FtGF, FtGG, FtGL and FtGB was used for continuous propagation of CyHV-2 with high viral titer of $10^{7.8}$±0.26 $TCID_{50}$/mL, $10^{7.375}$±0.35 $TCID_{50}$/mL, $10^{4.55}$±0.070 $TCID_{50}$/mL and $10^{6.45}$±0.070 $TCID_{50}$/mL respectively. CyHV-2 has been successfully propagated in FtGF, FtGG, FtGL and FtGB cell lines up to 20, 20, 10 and 8 passages respectively. An experimental infection of naive goldfish using supernatant from infected FtGF cells caused 100% mortality and CyHV-2 infection in the challenged fish was confirmed by the amplification of DNA polymerase gene, histopathology and transmission electron microscopy. By TEM analysis we could observe 170 -180 nm in diameter viral structure from the experimental infected fish tissues and CyHV-2 infected FtGF cell.

2.4. Discussion

In recent years, different cell lines from different tissues of *C. auratus* are developed (Li et al., 2021; Swaminathan, Raj et al., 2021) and fascinated significant attention as a means of viral disease diagnosis and their management in *C. auratus*, one of the highly traded

ornamental fish. Very fewer *C. auratus* cell lines had been used for the purpose and tested for their permissibility in sustainable isolation of CyHV-2 (Ito & Ototake, 2013; Shibata et al., 2015) and no cell lines from *C. auratus* was available in India. So, finfish virologist will still have to rely on available cell lines for the propagation of CyHV-2 from the suspected samples.

Here we reported the establishment of a novel and highly permissive cell line from fin, gill, liver and brain tissue of *C. auratus*, denoted as FtGF, FtGG, FtGL and FtGB that will consistently propagate CyHV-2. These cell lines maintained stable growth for 30–35 passages, over a period of two years and cell grew well in L-15 medium supplemented with 10% FBS at an incubation temperature of 28 °C. L-15 was the cell culture medium used by several investigators to establish other *C. auratus* cell lines (Yan et al., 2011) whereas M199 medium was used by (Jing et al., 2016, 2017). Further these cell lines exhibited a modal chromosome number of 100. It has been reported by several other researchers that the modal chromosome number of cell lines of *C. auratus* remained diploid (Yan et al., 2011). It was suggested that the modal chromosomal count of *C. auratus* will vary from 96 to 104 within the same individual (Ohno & Atkin, 1966) may be due to post-zygotic chromosomal rearrangements occur in the *C. auratus*. Cryopreservation of cell line in LN_2 is necessary for long-term storage for further usage. The viability of cryopreservation of FtGF, FtGG, FtGL and FtGB cells were confirmed, with recovery of up to 83%, 82%, 72% and 70% respectively after thawing. A sequence analysis of 16S rRNA confirmed that all the three cell lines were definitely derived from *C. auratus*.

The goldfish fin (GFF) cell line was used for in vitro propagation of CyHV-2, but the yield of virus was very low, with a titer value of $10^{2.0}$ $TCID_{50}$/mL (Ito & Ototake, 2013). Whereas, Ma et al. (2015) reported CyHV-2 titer was $10^{7.5\pm0.37}$ $TCID_{50}$/mL in the GiCB cell line developed from brain tissue of *C. gibelio* and has been continuously passaged over 50 times. At the same time, GFSe and GFKf cell lines developed from the snout and kidney tissue of the *C. auratus*, respectively, yielded infectious pancreatic necrosis virus (IPNV) titers of $10^{6.8}$ and $10^{5.9}$ $TCID_{50}$/mL, respectively (Jing et al., 2016). In the present study, we isolated CyHV-2 from infected *C. auratus* using highly permissible cell lines including FtGF, FtGG, FtGL and FtGB developed from *C. auratus* with good CyHV-2 titre of $10^{7.8\ \pm0.26}$, $10^{7.375\ \pm0.035}$, $10^{\ 4.55\pm0.070}$ and $10^{\ 6.45\pm0.070}$ $TCID_{50}$ /mL respectively. These results suggested that the cell lines developed from different organs and tissues of a host species differ in their susceptibility and yield to infection by fish viruses. Hence the

In our previous study we have observed that CyHV-2 can infect gills, liver, brain and eye in *C. auratus* (Sahoo et al., 2016). So, it is obvious that the establishment of a cell line from these target tissues would be useful in the propagation of CyHV-2 in high titre for vaccine development and rapid diagnostic kits for this disease.

2.5. Conclusion

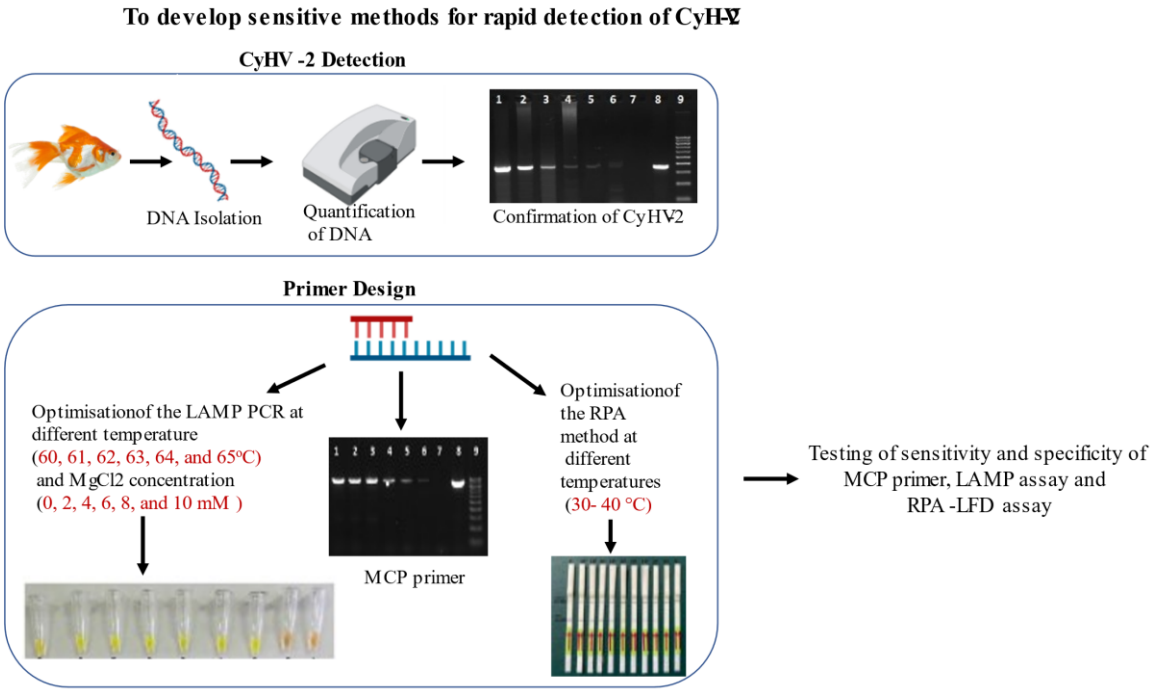
In conclusion, the newly established cell lines FtGF, FtGG, FtGB and FtGL can be used for CyHV-2 propagation. The cell lines FtGF and FtGG are highly susceptible to CyHV-2 culture *in vitro*. The newly developed cell lines would play a crucial role in future research on CyHV-2, including studying the molecular pathogenesis of GFHNV disease and development of strategies for the prevention and control of the disease in the country. The new FtGF (accession number; NRFC058 and FtGG (waiting for accession number) cell lines are deposited in the National Repository of Fish Cell Line (NRFC) (the largest fish cell line repository), ICAR National Bureau of Fish Genetic Resources, India for further dissemination to scientists for carrying out research in developing CyHV-2 management.

Chapter 3: To develop sensitive methods for rapid detection of CyHV-2

3.1. Introduction

The screening and primary level diagnosis of CyHV-2 infection are done by clinical signs and post mortem lesions. The major challenge in diagnosis of CyHV-2 is posed by the latency of virus in apparently healthy fish, because latently infected animals may carry the viral genome without producing infectious particles so that false-negative reactions are possible in cell culture or immunology-based diagnostic methods. Various molecular and immunological methods such as polymerase chain reaction (PCR) (Jeffery et al., 2007; Waltzek et al., 2009; Xu et al., 2013), real-time PCR (Goodwin et al., 2006b), loop-mediated isothermal amplification (LAMP) assay (He et al., 2013; Zhu et al., 2015), indirect immunofluorescence antibody test (Nanjo et al., 2017) and immuno-histochemistry assay (Kong et al., 2017). In the present work, we have developed a sensitive polymerase chain reaction (PCR), Loop mediated isothermal amplification (LAMP) assay and Recombinase polymerase amplification (RPA) and Lateral flow dipstick assay based on major capsid protein (MCP) gene of CyHV-2. The sensitivity and specificity of the developed assays were compared with the available PCR assay.

3.2. Methodology


To develop sensitive methods for rapid detection of CyHV-2
CyHV -2 Detection
DNA Isolation
Quantification of DNA
Confirmation of CyHV-2
Primer Design
Optimisationof the LAMP PCR at different temperature (60, 61, 62, 63, 64, and 65ºC) and MgCl2 concentration (0, 2, 4, 6, 8, and 10 mM)
MCP primer
Optimisationof the RPA method at different temperatures (30- 40 °C)
Testing of sensitivity and specificity of MCP primer, LAMP assay and RPA -LFD assay

3.3. Results

This is the first study where MCP gene primers were constructed for detecting CyHV-2. The LAMP assay was optimized at varying temperatures, where the amplification of LAMP product

was achieved at 8 mM $MgCl_2$ concentration and at a temperature of 60 ºC for 1 h. In case of RPA assay, the sensitive detection of CyHV-2 was attained at 36 ºC. The assays were highly specific for CyHV-2 and did not amplify the nucleic acids of other fish pathogens tested, namely spring viraemia of carp virus (SVCV), Cyprinid herpesvirus-3 (CyHV-3), and viral nervous necrosis virus (VNNV), *Aeromonas hydrophila, A. veronii, A. caviae, Edwardsiella tarda, Vibrio anguillarum, V. parahaemolyticus, V. harveyi* and *Proteus hauseri*. The MCP primer could detect 100 copies of the plasmid construct containing 942 bp fragment of MCP gene of CyHV-2. Among the other two assays developed, LAMP was capable of detecting 10 copies of the MCP plasmid construct while RPA - LFD could detect only 100 copies. The developed LAMP and RPA-LFD assays are simple, economical, rapid and reliable method for detection of CyHV-2 infection and can also be used in field conditions.

3.4. Discussion

Several molecular detection methods with high specificity and sensitivity including real-time PCR and PCR have been developed for detection of CyHV-2 (Goodwin et al., 2006a, Goodwin et al., 2006b, Waltzek et al., 2009). A rapid and highly sensitive diagnostic method would help in the early detection of CyHV-2 in goldfish populations and slow its spread. As major capsid protein, pORF92 is an immunogenic protein that plays a vital role in CyHV-2 infection, ORF92 was employed for the detection of CyHV-2 and was used as a marker gene for the development of LAMP and RPA-LFA for CyHV-2 diagnosis in this study.

In this study, the newly developed MCP PCR could amplify 100 copies using 10 fold serially diluted plasmid construct containing CyHV-2 MCP gene fragment as a standard. The sensitivity limit of the newly designed PCR primer was equal to the sensitivity of PCR based on DNA polymerase gene (Jeffery et al., 2007) in detecting CyHV-2 by PCR, whereas when compared to the sensitivity of the helicase gene primers (Goodwin et al., 2006a; Waltzek et al., 2009; Xu et al., 2013), the new MCP primer could detect lesser copy number of CyHV-2. Therefore, the newly designed MCP primers in this study provide alternate primers for detecting CyHV-2 infection in goldfish.

In our study, the detection limit of the LAMP assay was 10 copies of the recombinant 1 plasmid construct with the 942 bp MCP gene of CyHV-2. The results attained by LAMP ga was in agreement with previous report, where 10 copies of plasmid construct of intercapsomeric triplex protein gene of CyHV-2 (Liang et al., 2014) and 100 copies of pMD-hel plasmid of helicase gene of CyHV-2 (Zhu et al., 2015). RPA is less time consuming than the LAMP assay (Dai et al., 2019). An RPA primer targeting ORF 72 of CyHV-2 were

developed by Wang et al. in 2018. CyHV-3 detection were carried out using RPA primers of MCP by Prescott et al. (2016). Since, there has not been any RPA primers and probes developed for MCP gene of CyHV-2, this study has successfully developed a suitable assay for detecting CyHV-2.

3.5. Conclusion

The international trade of goldfish could facilitate the spread of CyHV-2 and non-availability of rapid detection methods will potentially put the native fish population at risk and cause disease out breaks. The LAMP and RPA-LFD assay developed in this study, based on the MCP gene of CyHV-2 has immense potential for rapid and sensitive diagnosis of CyHV-2 in field. These techniques are promising tool in aquaculture for field diagnosis, where expensive instrument or trained personnel or complicated procedures are barely needed. Furthermore, the new PCR method, LAMP and RPA-LFD assay will also serve as an essential tool for the development of potential disease control strategies viz., vaccine production and other future studies of CyHV-2 infection in fish in India.

Chapter 4: To develop inactivated vaccines to combat the spread of CyHV-2 in goldfish.

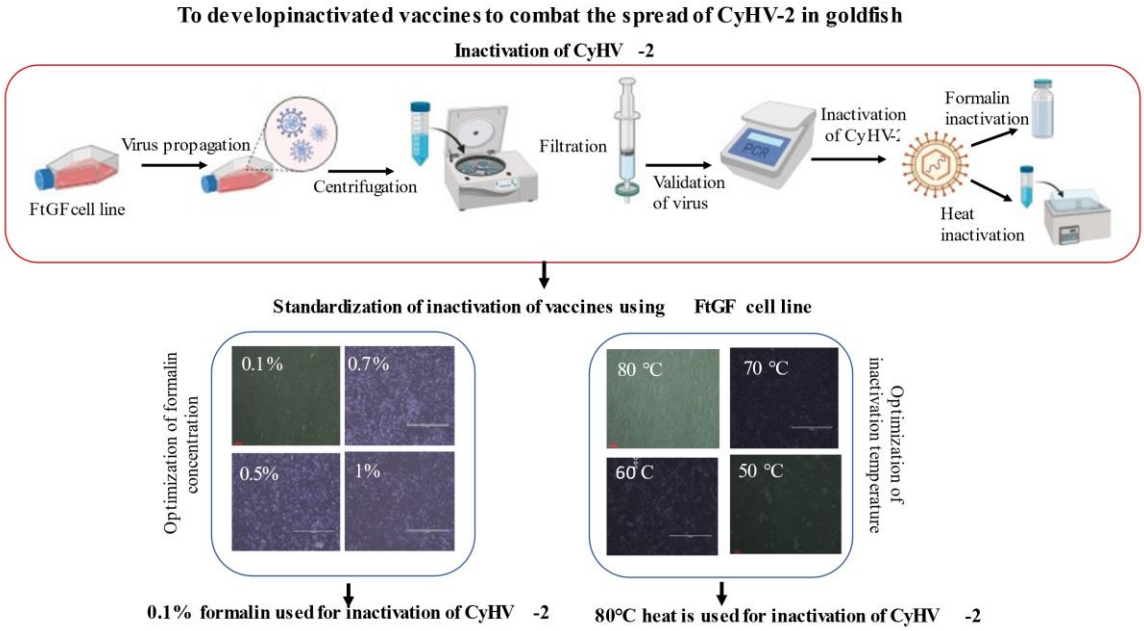
4.1. Introduction

Vaccination is being considered as the most preventive method for the bacterial and viral diseases. Progress in vaccine development have been from producing inactivated vaccines to the new generation vaccines (recombinant vaccines, DNA/ RNA vaccines, subunit vaccines, peptide vaccines, nano vaccines, reverse vaccinology derived vaccines) (Ma et al., 2019). The inactivated vaccines have paved way for a wider approach in development of commercial vaccines (Newman, 1993). For viral and parasitic diseases, vaccine development has proven more difficult. Traditional vaccines, that are whole killed, live attenuated, and subunit ones, have worked to some extent (Leong, 1993), but they each have particular safety, environmental or economic disadvantages.

Till date two types of inactivated CyHV-2 vaccines, i.e, formalin inactivated vaccine (Ito & Ototake, 2013) and β-propiolactone inactivated vaccine (Yan et al., 2020; Zhang et al., 2016) are produced in China. In 2020, Yan et al. studied the immunological activity of β-propiolactone inactivated vaccine when coupled with adjuvants such as β-glucan, DTT, scopolamine and anisodamine where he has found that the vaccines mixed with anisodamine, β-glucan and scopolamine gave more protection to gibel carps (*Carassius gibelio*). In China, a recombinant subunit CyHV-2 vaccine for the region ORF 25 was prepared in an expression system *Pichia pastoris* (Zhou et al., 2015). The DNA vaccines for CyHV-2 were studied using the live baculovirus vector or plasmid with or without adjuvant (Li et al., 2019; Huo et al., 2020). Several recombinant proteins (ORF16, tORF25-2, tORF64, and tORF146) were found to be immunogenic and a DNA vaccine, ORF25 shown an up regulation of the genes IL 1β, MHC-I, TF-A and C3 (Yuan et al., 2020). An oral yeast vaccination for CyHV-2 were developed in China by Dong et al which produced a strong immune response (Dong et al., 2022).

In our study we have prepared a formalin and heat inactivated CyHV-2 vaccine using the CyHV-2 supernatant collected from a highly species specific FtGF cell line (Dharmaratnam et al., 2020) containing a high viral concentration $10^{7.8}$ TCID50/mL. We have determined the effect of various concentrations of formalin (1%, 0.7%, 0.5%, 0.3% and 0.1%) inactivated CyHV-2 in FtGF cell lines. The various temperatures (40 °C, 50 °C, 60 °C, 70 °C, and 80 °C) were used for inactivating CyHV-2 and the temperature at which they were inactivated were analyzed using FtGF cell line.

4.2. Methodology



4.3. Results

Until now no proper prophylactic measure or treatment is available for CyHV-2 infection in goldfish. Hence, in our study we used FtGF cell line for propagation of virus having the titer of the CyHV-2 inoculum of $10^{7.8}$ $TCID_{50}$/mL. The complete inactivation of CyHV-2 was determined based on the morphological changes observed in FtGF. The CyHV-2 inoculam inactivated by 0.1% formalin for 2 days and the CyHV-2 inoculum inactivated at 80 °C for 1 hr did not show any cytopathic effect in the FtGF cell line after five blind passages. Hence, the 0.1% formalin inactivated CyHV-2 vaccine and heat-inactivated CyHV-2 vaccine developed at 80 °C was further used for immunization trials in goldfish.

4.4. Discussion

The main aim of vaccine is to develop an immunological memory, thus boosting up the adaptive immune response (McHeyzer-Williams & McHeyzer-Williams, 2005; Sallusto et al., 2010). Different types of vaccines are possible, i.e., inactivated vaccines either adjuvanted or not (Ito & Maeno, 2015; Yan et al., 2020; Zhang et al., 2016), live attenuated vaccines (Saito et al., 2022), recombinant sub-unit vaccines (Zhang et al., 2020), synthetic vaccines (peptide vaccines) or DNA vaccines (Huo et al., 2020; Yan et al., 2020). Compared to attenuated and DNA vaccines, inactivated vaccines are safe to be used as they are not infectious and do not cause horizontal transfer like live virus which would cause adverse

ecological effects to aquaculture environment unless well controlled. Inactivated vaccines can also induce strong immune response (Ma et al., 2019).

. In order to find remedy for the spread of CyHV-2, the attempt of producing vaccines started from 2013 (Ito et al., 2013). The formalin inactivated CyHV-2 vaccine provided a protection rate of 50% for *C. auratus* and further studies by the same team led to a booster dose vaccine which could attain a relative percentage survival (RPS) value of 63.6% (Ito & Maeno, 2014). Later, β-propiolactone inactivated vaccine could produce a relative percentage survival (RPS) value of 71.4% in *C. gibelio* (Zhang et al., 2016). In our study we have optimised the concentration of formalin and temperature required to denature the virus and this was studied using the cell line FtGF. Till date there have not been any standardization studies carried out to determine the virus inactivation. Here, we have seen that among all the concentrations (1%, 0.7%, 0.5%, 0.3% and 0.1%) of formalin used for inactivation, the best results were seen when the virus were inactivated at a concentration of 0.1% formalin incubated for 1 day. The heat inactivation temperature was optimized to 80 °C incubated for 45 minutes, where we could find that the FtGF cells had no morphological changes when inoculated with this.

4.5. Conclusion

Thus, we were able to produce a formalin and heat inactivated vaccine for CyHV-2. The vaccine studies in FtGF shown that the 0.1% formalin and 80 °C inactivated the CyHV-2 and five serial passages of the inactivated material confirmed absence of live virus in the inactivated harvest. Further immunological studies were carried out with these inactivated CyHV-2 vaccine.

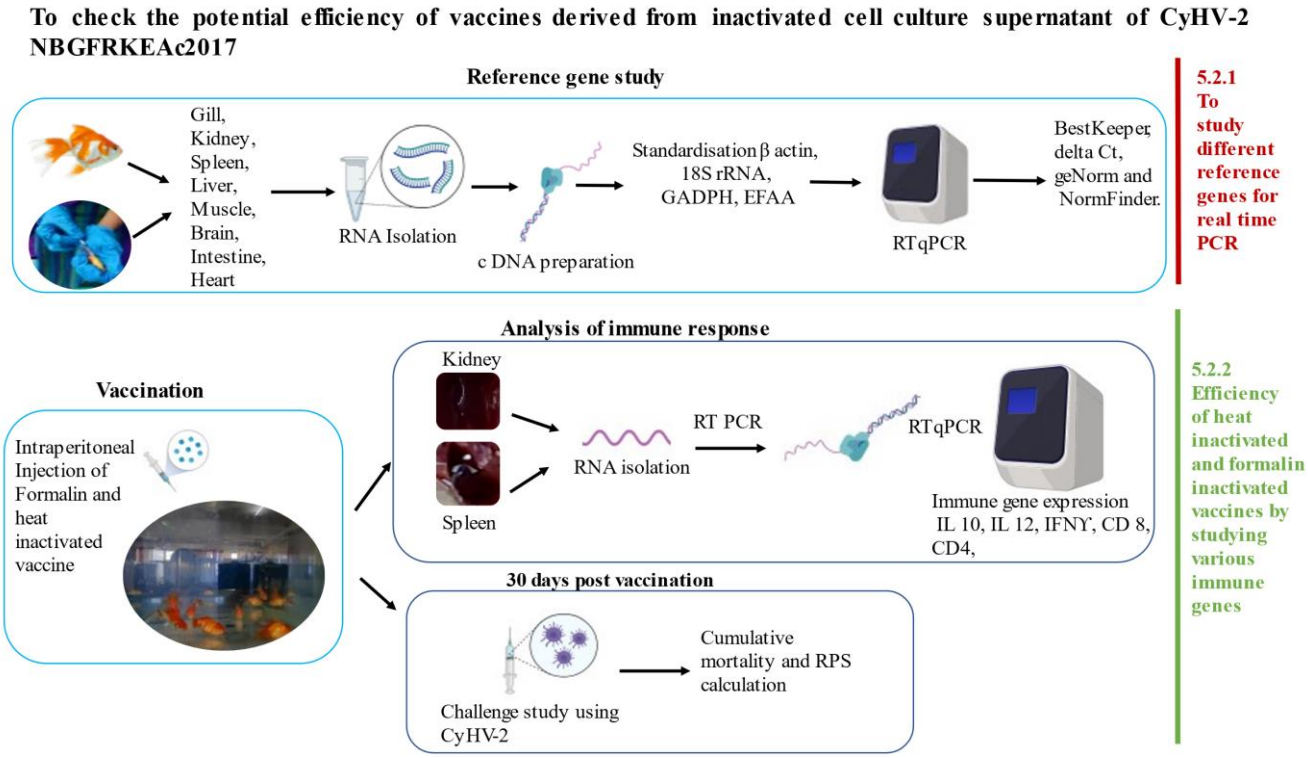
Chapter 5: To check the potential efficiency of vaccines derived from inactivated cell culture supernatant of CyHV-2 NBGFRKEAc2017

5.1. Introduction

The host system has developed its own sophisticated mechanisms to fight the viral infections and depending on the immune status of the host, the severity of the diseases varies (Vossen et al., 2002). The expression of the various immune genes at different intervals by the administration of vaccines are well studied using a quantitative real time polymerase chain reaction (RTqPCR). In aquaculture, the RTqPCR is used for disease diagnosis, immunological studies, developmental stage studies, environmental and stress related studies (Faheem et al., 2018; Rojas-Hernandez et al., 2019; Wang, Wen, et al., 2018).

To date, no reference genes have been screened in goldfish (*Carassius auratus*) for studying the Cyprinid herpesvirus 2 (CyHV-2) infection. For our study, we have evaluated four genes like glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-Actin (ACTB), 18S ribosomal RNA (18S) and elongation factor 1 alpha ($EF1A_A$). These four reference genes are the classical reference genes and are being taken based on their use as potential reference genes in fish based on the previous studies (Faheem et al., 2018; Wens et al., 2018).

5.2. Methodology


To check the potential efficiency of vaccines derived from inactivated cell culture supernatant of CyHV-2 NBGFRKEAc2017
Reference gene study
Gill, Kidney, Spleen, Liver, Muscle, Brain, Intestine, Heart
RNA Isolation
c DNA preparation
Standardisation β actin, 18S rRNA, GADPH, EFAA
RTqPCR
BestKeeper, delta Ct, geNorm and NormFinder.
5.2.1 To study different reference genes for real time PCR
Analysis of immune response
Vaccination
Intraperitoneal Injection of Formalin and heat inactivated vaccine
Kidney
Spleen
RNA isolation
RT PCR
RTqPCR
Immune gene expression IL 10, IL 12, IFNϒ, CD 8, CD4,
30 days post vaccination
Challenge study using CyHV-2
Cumulative mortality and RPS calculation
5.2.2 Efficiency of heat inactivated and formalin inactivated vaccines by studying various immune genes

5.3. Results

The present study has focused in the development of an inactivated vaccine for CyHV-2 and to check the immunogenicity of the vaccine in host. The accuracy of quantitative real time PCR (RTqPCR) can be attained only when a suitable reference gene is used. In this study, we have examined the potential of four different reference genes including β-Actin (ACTB), 18S ribosomal RNA (18S), glyceraldehyde-3P-dehydrogenase (GAPDH), and elongation factor 1 alpha ($EF1A_A$) in seven different tissues including gill, liver, kidney, spleen, heart, muscle and intestine of *C. auratus*. The housekeeping genes were analysed from healthy fish and in CyHV-2 challenged fish. The expression levels of the housekeeping genes were then compared and evaluated with the RefFinder web tool which analyses results using four different algorithms – BestKeeper, delta Ct, geNorm and NormFinder. ACTB was given as a stable gene by geNorm analysis in both healthy and challenged fish. $EF1A_A$ was also ranked to be the best gene in healthy fish by BestKeeper and geNorm analysis. The delta Ct and NormFinder algorithm have found 18S to be a stable gene in healthy fish but 18S was given to be least expressed in challenged fish. Also, in CyHV-2 challenged fish, $EF1A_A$ was identified as the best gene by all the three analyses.

The goldfishes were intraperitonially injected with vaccines and various immune gene response were studied for a period of 30 days. Subsequently, the kidney and spleen tissues were sampled at various time points post-vaccination (6^{th} hr, 2^{nd} day, 4^{th} day, 6^{th} day, 10^{th} day, 16^{th} day, and 30^{th} day) to evaluate the expression of immune genes (IL-12, IL-10, IFN-γ, CD8, and CD4). For both the formalin and heat inactivated CyHV-2, a significant up regulation was noticed for the genes CD8, GINF, IL-12 by the 6^{th} h itself whereas for the genes IL-10 and CD4, a significant up regulation was observed in the 6^{th} h for the heat inactivated virus. The experimental fish were challenged intraperitonially with CyHV-2 of concentration $10^{7.8}$ $TCID_{50}$/mL after 30 days of post vaccination. A significant difference in cumulative mortality rate were observed for the vaccinated fishes from the unvaccinated fishes. The relative survival rate for heat immunized fishes were 83.34 %. and formalin immunized fishes were 74.03 %. Our results have proven that both the vaccines were efficient but heat inactivated CyHV-2 vaccines shown more triggering immune gene expression than the formalin inactivated vaccines. The development and further enhanced studies for these vaccines will lead to a promising vaccine for CyHV-2 viral disease.

5.4. Discussion

During the invasion of the pathogen, they try to make their own identity in the host by regulating and modulating the cellular processes and making some changes in the normal biological pathways (Watson et al., 2007). Hence there is a need to study the stability of various reference genes. So, we carried out a study, where reference genes such as ACTB, $EF1A_A$, GADPH and 18S are studied for *C. auratus* before and after infection with the CyHV-2 virus for the first time. We have used the RefFinder algorithm, which is online software that uses four different analysis methods (comparative ΔCt method, geNorm, BestKeeper, NormFinder). This algorithm is being widely used to identify suitable reference genes. 18S gene and $EF1A_A$ genes can be effectively used for the normalization of RTqPCR in healthy fish, whereas post-infection, inferred $EF1A_A$ and ACTB to be the best endogenous gene and 18S as the least stable gene. In our study, we have attained a lower Ct value for ACTB in both healthy and challenged *C. auratus*, which signifies its high expression and this was used in our further vaccine study. Moreover, the geNorm analysis showed that ACTB to be a stable gene for both healthy and challenged *C. auratus*. ACTB has been selected as the best endogenous gene in different fish like *Lates calcarifer*, Asian seabass (Paria et al., 2016), *Paralichthys olivaceus* , Japanese flounder.

There are not much immune genes studied for the developed CyHV-2 vaccines. Only a few genes such as IL-2 and IFN-γ2 (Yan et al., 2020) and IL 1β, MHC-I, TF-A and C3 (Yuan et al., 2020) were seem to be upregulated after the administration of β-propiolactone inactivated vaccine and DNA vaccines. The kidney and spleen serve as a major lymphoid organ in teleost and evaluating immune gene expression on these organs can provide valuable insights into the performance of the vaccine (Press & Evensen, 1999).

Expression pattern of five immune genes (IL-12, IL-10, IFN-ϒ, CD8, and CD4) were evaluated in the present study using RTqPCR. For both the vaccines, the IL-12 is activated in the 6^{th} h itself when compared to the control cells. IL-12 is one among the proinflammatory molecule released as a primary response of the cell to eliminate the pathogen and ultimately activates the antigen presenting cells to produce adaptive immunity (Smith & Khanna, 2013). IFN II cytokine is studied to act as a regulatory gene in the host innate and adaptive immune responses (Schoenborn & Wilson, 2007). In heat inactivated vaccines, the level of IFN is upregulated by 6^{th} h in kidney and spleen, reaching its peak on 16^{th} day. The same phenomenon was observed for formalin inactivated vaccines.

We could find that the IFN-ϒ expression to be lowered by the 30th day, at the same time the IL-10 level is increased by the 30th day. This holds up the theory that the IFN-ϒ production is suppressed by the production of IL-10 (Ding et al., 2003). This shows that there has been a switching of immune system to a Th2 system from a Th1 response (Berger, 2000). The CD8 cells engage in destruction of these antigens and forms a memory T cells. CD8 plays a major role in the cell mediated responses for elimination of virus (Adedeji et al., 2012). In our study for both the vaccines, the expression of CD8 is significantly over expressed than the control fishes by the 6th h post vaccination in both kidney and spleen tissues. For the formalin inactivated vaccines, CD4 gene have come into immediate response soon after the down regulation of CD8 gene in kidney and then in spleen. In case of heat inactivated vaccines, we have noticed the elevated expression of CD4 in spleen tissue by 6th h and was seen to be up regulated till the 16th day post vaccination. This might be because the CD4 cells seems to play a major role in the early activation of CD8 T cells, thus leading to the development of B-cell (Ziegler, 2016). The vaccine studies for CyHV-2 were first carried out by Ito & Ototake in 2013 where they have developed a formalin-inactivated vaccine and that vaccine could offer 63.6% of RPS. Similarly, a β-propiolactone inactivated vaccine of CyHV-2 by Zhang et al. (2016) showed an RPS of 71.4%. However, in the present study, the formalin-inactivated vaccine could provide a high protection rate of 74.03% and 83.34% for heat inactivated CyHV-2 in *C. auratus* against CyHV-2. The results suggest that a single dose of inactivated viral vaccine can protect the host against the CyHV-2 virus infection.

5.5. Conclusion

In conclusion, both the formalin and heat inactivated CyHV-2 vaccines are highly effective in preventing CyHV-2 infection in *C. auratus*. Moreover, the vaccine could elicit an immune response in the vaccinated fish. Among the two CyHV-2 vaccines developed, the heat-inactivated vaccine offered better protection against CyHV- 2 infection in *C. auratus*. However, further field level studies are required to explore the commercial potential and economic feasibility of these vaccines.

SUMMARY AND CONCLUSION

Due to the enormous trade of ornamental fishes, they are constrained to various pathogens of which the viral disease poses serious economic loss. The CyHV-2 virus caused extensive mass mortality of *C. auratus* in India from 2016, congruent with the reports from several other countries. The ability of the virus to exist in latent stages poses a great threat to the effective diagnosis of CyHV-2 infections. Identification of the virus in the prior level will definitely help in prevention and spread of the viral disease. Decades of research were conducted in developing cell lines for propagating CyHV-2 and among those only the cell line GiCB, were found to attain a higher titre value of $10^{7.5\pm0.37}$ $TCID_{50}$/mL. In spite of all these cell lines, none of these cell lines were available in India. Consequently, there urged the development of cell line from India in order to detect the CyHV-2 and to take the further results to a higher level. Till today various approaches in vaccines are being developed, however the developed vaccines studied are not commercialized. Standing against various vaccine studies, only a few have reported the immunological response of vaccine.

Salient achievements from this study

- The present study proposes the development of cell lines from various tissues (fin, gill, brain and liver) of goldfish which were then characterized and cryopreserved.
- The developed cell lines FtGF, FtGG, FtGB and FtGL were successful in propagation of CyHV-2 giving viral titers of $10^{7.8}\pm0.26$ $TCID_{50}$/mL, $10^{7.375}\pm0.35$ $TCID_{50}$/mL, $10^{6.45}\pm0.070$ $TCID_{50}$/mL and $10^{4.55}\pm0.070$ $TCID_{50}$/mL respectively.
- All the results provide solid evidence that the FtGF and FtGG cell line is highly permissive for the isolation and propagation of CyHV-2.
- The TEM analysis of the purified CyHV-2 gave a size of 170–180 nm in diameter which was relevant to the earlier observed structure
- The present study proposes the development of new PCR primers for the amplification of major capsid protein (MCP) gene. The MCP is encoded by ORF92 gene which is used as capture antigen in immunological studies and vaccine development
- The present study also focused to develop LAMP and RPA primers and probes targeting the MCP gene and shown high sensitivity, specificity, rapidity, reproducibility and applicability. The LAMP could detect 10 copies of virus whereas MCP primer and RPA detected upto 100 copies of virus.

- This can be suggested as a promising tool to the farmers for field diagnosis in aquaculture units where trained personnel or expensive instrument or complicated procedures are hardly needed and hence easily accessible to the masses
- From our study we suggested the expression stability of the reference genes – ACTB, $EF1A_A$, GADPH and 18S in various tissues of healthy and CyHV-2 challenged fish.
- We have extended our reference gene study in seven different tissues like gill, kidney, liver, spleen, muscle, intestine and heart. The results suggests that the expression of ACTB, $EF1A_A$, GADPH and 18S are tissue specific.
- As vaccination is an easy, and effective method of protecting fish from diseases, this study proposes the development of two types of inactivated vaccines – heat and formalin inactivated vaccines.
- This is the first report where heat inactivated CyHV-2 vaccines are produced. We could observe that the RPS values (74.03% & 83.43%) produced by formalin and heat inactivated vaccines were observed to be more than the previously produced CyHV-2 vaccines
- This is the first report where the immune genes such as IL-12, IL-10, CD8, CD4 and IFN-ϒ were studied in kidney and spleen tissues
- The genes such as IL-12, IFN-ϒ and CD8 were elevated by the 6^{th} h in formalin inactivated vaccine, whereas all the five genes were up regulated in heat inactivated vaccines. Thus, it implies both vaccines can produce both innate and adaptive immune response.
- In heat inactivated vaccines, we could observe the upregulation of most of the immune genes in both kidney and spleen tissues by 6^{th} h itself. Thus, pointed out that heat inactivated vaccines are better than formalin inactivated vaccines in raising the immune response.

Conclusion

The newly developed cell lines FtGF, FtGG provide solid evidence that these cell lines are highly permissive for the propagation of CyHV-2 and can be used further downstream applications including development of vaccines and diagnostic kits. The newly developed diagnostic techniques like LAMP and RPA-LFD can be

recommended as a rapid point-of-care diagnostic kit at the field level without the help of sophisticated equipments. The transcription variation data obtained from this study for the reference genes including ACTB, $EF1A_A$, GADPH and 18S in healthy and CyHV-2 infected goldfish can be further used in gene expression studies and immune response studies to develop better health strategies in the disease management of *C. auratus* aquaculture. The efficacy of the currently developed vaccines (heat and formalin inactivated vaccines) studied by immune gene expression revealed that both the vaccine could elicit innate and adaptive immune response. The RPS obtained for heat inactivated vaccine (83.34%) was higher than the formalin inactivated vaccines (74.03%) which infers that the heat inactivated vaccine were more affective than the formalin inactivated vaccine. However, additional field-level research is necessary to observe the marketability and viability of these vaccinations from an economic standpoint.

Reference

Adamek, M., Hellmann, J., Jung-Schroers, V., Teitge, F., & Steinhagen, D. (2018). CyHV-2 transmission in traded goldfish stocks in Germany—A case study. *Journal of Fish Diseases*, *41*(2), 401–404. https://doi.org/10.1111/jfd.12734

Adedeji, B. O., Onianwa, O., Okerentugba, P. O., & Okonko, I. O. (2012). Immune response of fish to viral infection. *Nature and Science*, *10*, 70–76.

Berger, A. (2000). Science commentary: Th1 and Th2 responses: What are they? *British Medical Journal*, *321*(7258), 424. https://doi.org/10.1136/bmj.321.7258.424

Bernoth, E. M., & Crane, M. S. J. (1995). Viral diseases of aquarium fish. *Seminars in Avian and Exotic Pet Medicine*, *4*(2), 103–110. https://doi.org/10.1016/S1055-937X(05)80046-5

BERRY, E. S., SHEA, T. B., & GABLIKS, J. (1983). Two iridovirus isolates from Carassius auratus (L.). *Journal of Fish Diseases*, *6*(6), 501–510. https://doi.org/10.1111/j.1365-2761.1983.tb00104.x

Biondo, M. V., & Burki, R. P. (2020). A systematic review of the ornamental fish trade with emphasis on coral reef fishes—an impossible task. *Animals*, *10*(11), 1–21. https://doi.org/10.3390/ani10112014

Boitard, P. M., Baud, M., Labrut, S., de Boisséson, C., Jamin, M., & Bigarré, L. (2016). First detection of Cyprinid Herpesvirus 2 (CyHV-2) in goldfish (Carassius auratus) in France. *Journal of Fish Diseases*, *39*(6), 673–680. https://doi.org/10.1111/jfd.12400

Cao, Z., Liu, S., Nan, H., Zhao, K., Xu, X., Wang, G., Ji, H., & Chen, H. (2019). Immersion immunization with recombinant baculoviruses displaying cyprinid herpesvirus 2 membrane proteins induced protective immunity in gibel carp. *Fish and Shellfish Immunology*, *93*(March), 879–887. https://doi.org/10.1016/j.fsi.2019.08.036

Chang, P. H., Lee, S. H., Chiang, H. C., & Jong, M. H. (1999). Epizootic of herpes-like virus infection in goldfish, Carassius auratus in Taiwan. *Fish Pathology*, *34*(4), 209–210. https://doi.org/10.3147/jsfp.34.209

Costes, B., Fournier, G., Michel, B., Delforge, C., Raj, V. S., Dewals, B., Gillet, L., Drion, P., Body, A., Schynts, F., Lieffrig, F., & Vanderplasschen, A. (2008). Cloning of the Koi Herpesvirus Genome as an Infectious Bacterial Artificial Chromosome Demonstrates That Disruption of the Thymidine Kinase Locus Induces Partial Attenuation in Cyprinus carpio koi . *Journal of Virology*, *82*(10), 4955–4964. https://doi.org/10.1128/jvi.00211-08

Dai, T., Yang, X., Hu, T., Jiao, B., Xu, Y., Zheng, X., & Shen, D. (2019). Comparative evaluation of a novel recombinase polymerase amplification-lateral flow dipstick (Rpa-lfd) assay, lamp, conventional pcr, and leaf-disc baiting methods for detection of phytophthora sojae. *Frontiers in Microbiology*, *10*(AUG), 1–10. https://doi.org/10.3389/fmicb.2019.01884

Daněk, T., Kalous, L., Veselý, T., Krásová, E., Reschová, S., Rylková, K., Kulich, P., Petrtýl, M., Pokorová, D., & Knytl, M. (2012). Massive mortality of Prussian carp Carassius gibelio in the upper Elbe basin associated with herpesviral hematopoietic necrosis (CyHV-2). *Diseases of Aquatic Organisms*, *102*(2), 87–95. https://doi.org/10.3354/dao02535

Davison, A. J., Eberle, R., Ehlers, B., Hayward, G. S., McGeoch, D. J., Minson, A. C., Pellett, P. E., Roizman, B., Studdert, M. J., & Thiry, E. (2009). The order Herpesvirales. *Archives of Virology*, *154*(1), 171–177. https://doi.org/10.1007/s00705-008-0278-4

Davison, A. J., Kurobe, T., Gatherer, D., Cunningham, C., Korf, I., Fukuda, H., Hedrick, R. P., & Waltzek, T. B. (2013). Comparative Genomics of Carp Herpesviruses. *Journal of Virology*, *87*(5), 2908–2922. https://doi.org/10.1128/jvi.03206-12

Dharmaratnam, A., Kumar, R., Valaparambil, B. S., Sood, N., Pradhan, P. K., Das, S., & Swaminathan, T. R. (2020). Establishment and characterization of fantail goldfish fin (FtGF) cell line from goldfish, Carassius auratus for in vitro propagation of Cyprinid herpes virus-2 (CyHV-2). *PeerJ*, *2020*(7). https://doi.org/10.7717/peerj.9373

Ding, Y., Chen, D., Tarcsafalvi, A., Su, R., Qin, L., & Bromberg, J. S. (2003). Suppressor of Cytokine Signaling 1 Inhibits IL-10-Mediated Immune Responses. *The Journal of Immunology*, *170*(3), 1383–1391. https://doi.org/10.4049/jimmunol.170.3.1383

Dong, Z. R., Mu, Q. J., Kong, W. G., Qin, D. C., Zhou, Y., Wang, X. Y., Cheng, G. F., Luo, Y. Z., Ai, T. S., & Xu, Z. (2022). Gut mucosal immune responses and protective efficacy of oral yeast Cyprinid herpesvirus 2 (CyHV-2) vaccine in Carassius auratus gibelio. *Frontiers in Immunology*, *13*(July), 1–15. https://doi.org/10.3389/fimmu.2022.932722

Doszpoly, A., Benko, M., Csaba, G., Dan, A., Lang, M., Harrach, B., & others. (2011). Introduction of the family Alloherpesviridae: the first molecular detection of herpesviruses of cyprinid fish in Hungary. *Magyar Allatorvosok Lapja*, *133*(3), 174–181.

Faheem, M., Jahan, N., Khaliq, S., & Lone, K. P. (2018). Validation of reference genes for expression analysis

in a teleost fish (Catla catla Hamilton) exposed to an endocrine-disrupting chemical, bisphenol-A. *Rendiconti Lincei. Scienze Fisiche e Naturali*, *29*(1), 13–22.

Fichi, G., Cardeti, G., Cocumelli, C., Vendramin, N., Toffan, A., Eleni, C., Siemoni, N., Fischetti, R., & Susini, F. (2013). Detection of Cyprinid herpesvirus 2 in association with an Aeromonas sobria infection of Carassius carassius (L.), in Italy. *Journal of Fish Diseases*, *36*(10), 823–830. https://doi.org/10.1111/jfd.12048

Ghosh, A., Mahapatra, B. K., & Datta, N. C. (2003). Ornamental fish farming-successful small scale aqua business in India. *Aquaculture Asia*, *8*(3), 14–16.

Giovannini, S., Bergmann, S. M., Keeling, C., Lany, C., Schütze, H., & Schmidt-Posthaus, H. (2016). Herpesviral Hematopoietic Necrosis in Goldfish in Switzerland: Early Lesions in Clinically Normal Goldfish (Carassius auratus). *Veterinary Pathology*, *53*(4), 847–852. https://doi.org/10.1177/0300985815614974

Goodwin, A. E., Khoo, L., LaPatra, S. E., Bonar, A., Key, D. W., Garner, M., Lee, M. V., & Hanson, L. (2006). Goldfish hematopoietic necrosis herpesvirus (cyprinid herpesvirus 2) in the USA: Molecular confirmation of isolates from diseased fish. *Journal of Aquatic Animal Health*, *18*(1), 11–18. https://doi.org/10.1577/H05-007.1

Goodwin, A. E., Merry, G. E., & Sadler, J. (2006). Detection of the herpesviral hematopoietic necrosis disease agent (Cyprinid herpesvirus 2) in moribund and healthy goldfish: Validation of a quantitative PCR diagnostic method. *Diseases of Aquatic Organisms*, *69*(2–3), 137–143. https://doi.org/10.3354/dao069137

Groff, J. M., LaPatra, S. E., Munn, R. J., & Zinkl, J. G. (1998). A viral epizootic in cultured populations of juvenile goldfish due to a putative herpesvirus etiology. *Journal of Veterinary Diagnostic Investigation*, *10*(4), 375–378. https://doi.org/10.1177/104063879801000415

He, J., Shi, X., Yu, L., Zheng, X., Lan, W., Jia, P., Wang, J., & Liu, H. (2013). Development and evaluation of a loop-mediated isothermal amplification assay for diagnosis of Cyprinid herpesvirus 2. *Journal of Virological Methods*, *194*(1–2), 206–210. https://doi.org/10.1016/j.jviromet.2013.08.028

Hedrick, R. P., Gilad, O., Yun, S., Spangenberg, J. V, Marty, G. D., Nordhausen, R. W., Kebus, M. J., Bercovier, H., & Eldar, A. (2000). A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp. *Journal of Aquatic Animal Health*, *12*(1), 44–57.

Hong, Y. J., Yu, Z. J., Zhou, L., & Gui, J. F. (2005). A population of red-transparent, triploid Carassius auratus. *Journal of Fish Biology*, *67*(4), 1139–1143. https://doi.org/10.1111/j.0022-1112.2005.00789.x

Huo, X., Fan, C., Ai, T., & Su, J. (2020). The combination of molecular adjuvant CCL35.2 and dna vaccine significantly enhances the immune protection of carassius auratus gibelio against cyhv-2 infection. *Vaccines*, *8*(4), 1–19. https://doi.org/10.3390/vaccines8040567

Ito, T., Kurita, J., & Haenen, O. L. M. (2017). Importation of CyHV-2-infected goldfish into the Netherlands. *Diseases of Aquatic Organisms*, *126*(1), 51–62. https://doi.org/10.3354/dao03157

Ito, T., Kurita, J., Ozaki, A., Sano, M., Fukuda, H., & Ototake, M. (2013). Growth of cyprinid herpesvirus 2

(CyHV-2) in cell culture and experimental infection of goldfish Carassius auratus. *Diseases of Aquatic Organisms*, *105*(3), 193–202. https://doi.org/10.3354/dao02627

Ito, T., & Maeno, Y. (2014). Susceptibility of Japanese Cyprininae fish species to cyprinid herpesvirus 2 (CyHV-2). *Veterinary Microbiology*, *169*(3–4), 128–134. https://doi.org/10.1016/j.vetmic.2014.01.002

Ito, T., & Maeno, Y. (2015). Effect of booster shot and investigation of vaccination efficacy period against herpesviral haematopoietic necrosis (HVHN) in goldfish Carassius auratus. *Veterinary Microbiology*, *175*(1), 139–144. https://doi.org/10.1016/j.vetmic.2014.11.005

Ito, T., & Ototake, M. (2013). Vaccination against cyprinid herpesvirus 2 (CyHV-2) infection in goldfish Carassius auratus. *Bulletin of the European Association of Fish Pathologists*, *33*(5), 158–164.

Jeffery, K. R., Bateman, K., Bayley, A., Feist, S. W., Hulland, J., Longshaw, C., Stone, D., Woolford, G., & Way, K. (2007). Isolation of a cyprinid herpesvirus 2 from goldfish, Carassius auratus (L.), in the UK. *Journal of Fish Diseases*, *30*(11), 649–656. https://doi.org/10.1111/j.1365-2761.2007.00847.x

Jiang, N., Xu, J., Ma, J., Fan, Y., Zhou, Y., Liu, W., & Zeng, L. (2015). Histopathology and ultrastructural pathology of cyprinid herpesvirus II (CyHV-2) infection in gibel carp, Carassius auratus gibelio. *Wuhan University Journal of Natural Sciences*, *20*(5), 413–420. https://doi.org/10.1007/s11859-015-1114-9

Jing, H., Gao, L., Zhang, M., Wang, N., Lin, X., Zhang, L., & Wu, S. (2016). Establishment from the snout and kidney of goldfish, Carassius auratus, of two new cell lines and their susceptibility to infectious pancreatic necrosis virus. *Fish Physiology and Biochemistry*, *42*(1), 303–311. https://doi.org/10.1007/s10695-015-0138-6

Jing, H., Lin, X., Xu, L., Gao, L., Zhang, M., Wang, N., & Wu, S. (2017). Establishment and characterization of a heart-derived cell line from goldfish (Carassius auratus). *Fish Physiology and Biochemistry*, *43*(4), 977–986. https://doi.org/10.1007/s10695-017-0345-4

JUNG, S. J., & MIYAZAKI, T. (1995). Herpesviral haematopoietic necrosis of goldfish, Carassius auratus (L.). *Journal of Fish Diseases*, *18*(3), 211–220. https://doi.org/10.1111/j.1365-2761.1995.tb00296.x

Kalayci, G., Ozkan, B., Pekmez, K., Kaplan, M., Mefut, A., & Anil Cagirgan, A. (2018). First detection of Cyprinid herpesvirus-2 (CyHV-2) followed by screening and monitoring studies in Goldfish (Carassius Auratus) in Turkey. *Bulletin of the European Association of Fish Pathologists*, *38*(4), 94–103.

Kibenge, F. S. B., Godoy, M. G., Fast, M., Workenhe, S., & Kibenge, M. J. T. (2012). Countermeasures against viral diseases of farmed fish. *Antiviral Research*, *95*(3), 257–281.

Kong, S. Y., Jiang, Y. S., Wang, Q., Lu, J. F., Xu, D., & Lu, L. Q. (2017). Detection methods of Cyprinid herpesvirus 2 infection in silver crucian carp (Carassius auratus gibelio) via a pORF72 monoclonal antibody. *Journal of Fish Diseases*, *40*(12), 1791–1798. https://doi.org/10.1111/jfd.12648

Kroeker, H. M. (1964). *The establishment and maintenance of goldfish cell lines in tissue culture*.

Lee, L. E. J., Caldwell, S. J., & Gibbons, J. (1997). Development of a cell line from skin of goldfish, Carassius auratus, and effects of ascorbic acid on collagen deposition. *The Histochemical Journal*, *29*(1), 31–43.

Leong, J. A. C. (1993). Molecular and biotechnological approaches to fish vaccines. *Current Opinion in Biotechnology*, *4*(3), 286–293. https://doi.org/10.1016/0958-1669(93)90097-G

Lepa, A., & Krzysztof Siwicki, A. (2012). Fish herpesvirus diseases: A short review of current knowledge. *Acta Veterinaria Brno*, *81*(4), 383–389. https://doi.org/10.2754/avb201281040383

Li, K., Yuan, R., Zhang, M., Zhang, T., Gu, Y., Zhou, Y., Dai, Y., Fang, P., Feng, Y., Hu, X., Cao, G., Xue, R., Chen, H., & Gong, C. (2019). Recombinant baculovirus BacCarassius-D4ORFs has potential as a live vector vaccine against CyHV-2. *Fish and Shellfish Immunology*, *92*(May), 101–110. https://doi.org/10.1016/j.fsi.2019.05.065

Li, L., Luo, Y., Gao, Z., Huang, J., Zheng, X., Nie, H., Zhang, J., Lin, L., & Yuan, J. (2015). Molecular characterisation and prevalence of a new genotype of cyprinid herpesvirus 2 in mainland China. *Canadian Journal of Microbiology*, *61*(6), 381–387. https://doi.org/10.1139/cjm-2014-0796

Li, N., Guo, L., & Guo, H. (2021). Establishment, characterization, and transfection potential of a new continuous fish cell line (CAM) derived from the muscle tissue of grass goldfish (Carassius auratus). *In Vitro Cellular \& Developmental Biology-Animal*, *57*(9), 912–931.

Li, S., Xie, H., Yan, Z., Li, B., Wu, P., Qian, X., Zhang, X., Wu, J., Liu, J., & Zhao, X. (2019). Development of a live vector vaccine against infectious hematopoietic necrosis virus in rainbow trout. *Fish and Shellfish Immunology*, *89*(February), 516–524. https://doi.org/10.1016/j.fsi.2019.04.024

Li, X., & Fukuda, H. (2003). In vitro culture of goldfish cell sensitive to goldfish herpesvirus. *Journal of Shanghai Fisheries University*, *12*(1), 12–18.

Liang, L. G., Xie, J., & Luo, D. (2014). Development of a rapid cyprinid herpesvirus 2 detection method by loop-mediated isothermal amplification. *Letters in Applied Microbiology*, *59*(4), 432–437. https://doi.org/10.1111/lam.12296

Liu, B., Zhou, Y., Li, K., Hu, X., Wang, C., Cao, G., Xue, R., & Gong, C. (2018). The complete genome of Cyprinid herpesvirus 2, a new strain isolated from Allogynogenetic crucian carp. *Virus Research*, *256*, 6–10. https://doi.org/10.1016/j.virusres.2018.07.016

Liu, C., Xin, N., Zhai, Y., Jiang, L., Zhai, J., Zhang, Q., & Qi, J. (2014). Reference gene selection for quantitative real-time RT-PCR normalization in the half-smooth tongue sole (Cynoglossus semilaevis) at different developmental stages, in various tissue types and on exposure to chemicals. *PLoS ONE*, *9*(3). https://doi.org/10.1371/journal.pone.0091715

Lu, J., Xu, D., & Lu, L. (2018). A novel cell line established from caudal fin tissue of Carassius auratus gibelio is susceptible to cyprinid herpesvirus 2 infection with the induction of apoptosis. *Virus Research*, *258*(June), 19–27. https://doi.org/10.1016/j.virusres.2018.09.010

Luo, Y. Z., Lin, L., Liu, Y., Wu, Z. X., Gu, Z. M., Li, L. J., & Yuan, J. F. (2013). Haematopoietic necrosis of cultured prussian carp, carassius gibelio (bloch), associated with cyprinid herpesvirus 2. *Journal of Fish Diseases*, *36*(12), 1035–1039. https://doi.org/10.1111/jfd.12110

Ma, J., Bruce, T. J., Jones, E. M., & Cain, K. D. (2019). A review of fish vaccine development strategies:

Conventional methods and modern biotechnological approaches. *Microorganisms*, *7*(11), 569.

Ma, J., Jiang, N., LaPatra, S. E., Jin, L., Xu, J., Fan, Y., Zhou, Y., & Zeng, L. (2015). Establishment of a novel and highly permissive cell line for the efficient replication of cyprinid herpesvirus 2 (CyHV-2). *Veterinary Microbiology*, *177*(3–4), 315–325. https://doi.org/10.1016/j.vetmic.2015.04.006

McGeoch, D. J., Rixon, F. J., & Davison, A. J. (2006). Topics in herpesvirus genomics and evolution. *Virus Research*, *117*(1), 90–104. https://doi.org/10.1016/j.virusres.2006.01.002

McHeyzer-Williams, L. J., & McHeyzer-Williams, M. G. (2005). Antigen-specific memory B cell development. *Annual Review of Immunology*, *23*(9), 487–513. https://doi.org/10.1146/annurev.immunol.23.021704.115732

Monticini, P. (2010). *The ornamental fish trade: production and commerce of ornamental fish: technical-managerial and legislative aspects*.

Nanjo, A., Shibata, T., Saito, M., Yoshii, K., Tanaka, M., Nakanishi, T., Fukuda, H., Sakamoto, T., Kato, G., & Sano, M. (2017). Susceptibility of isogeneic ginbuna Carassius auratus langsdorfii Temminck et Schlegel to cyprinid herpesvirus-2 (CyHV-2) as a model species. *Journal of Fish Diseases*, *40*(2), 157–168. https://doi.org/10.1111/jfd.12500

Ohno, S., & Atkin, N. B. (1966). Comparative DNA values and chromosome complements of eight species of fishes. *Chromosoma*, *18*(3), 455–466.

Olivotto, I., Planas, M., Simões, N., Holt, G. J., Avella, M. A., & Calado, R. (2011). Advances in Breeding and Rearing Marine Ornamentals. *Journal of the World Aquaculture Society*, *42*(2), 135–166. https://doi.org/10.1111/j.1749-7345.2011.00453.x

Panicz, R., Sadowski, J., & Eljasik, P. (2019). Detection of Cyprinid herpesvirus 2 (CyHV-2) in symptomatic ornamental types of goldfish (Carassius auratus) and asymptomatic common carp (Cyprinus carpio) reared in warm-water cage culture. *Aquaculture*, *504*(October 2018), 131–138. https://doi.org/10.1016/j.aquaculture.2019.01.065

Paria, A., Dong, J., Suresh Babu, P. P., Makesh, M., Chaudhari, A., Thirunavukkarasu, A. R., Purushothaman, C. S., & Rajendran, K. V. (2016). Evaluation of candidate reference genes for quantitative expression studies in asian seabass (Lates calcarifer) during ontogenesis and in tissues of healthy and infected fishes. *Indian Journal of Experimental Biology*, *54*(9), 597–605.

Prescott, M. A., Reed, A. N., Jin, L., & Pastey, M. K. (2016). Rapid Detection of Cyprinid Herpesvirus 3 in Latently Infected Koi by Recombinase Polymerase Amplification. *Journal of Aquatic Animal Health*, *28*(3), 173–180. https://doi.org/10.1080/08997659.2016.1185048

Press, C. M. L., & Evensen, O. (1999). The morphology of the immune system in teleost fishes. *Fish and Shellfish Immunology*, *9*(4), 309–318. https://doi.org/10.1006/fsim.1998.0181

Radonić, A., Thulke, S., Bae, H. G., Müller, M. A., Siegert, W., & Nitsche, A. (2005). Reference gene selection for quantitative real-time PCR analysis in virus infected cells: SARS corona virus, Yellow fever virus, Human Herpesvirus-6, Camelpox virus and Cytomegalovirus infections. *Virology Journal*, *2*(Cmv), 1–5.

https://doi.org/10.1186/1743-422X-2-7

Rani, P., Immanuel, S., & Kumar, N. R. (2014). *Ornamental Fish Exports from India Performance , Competitiveness and Ornamental Fish Exports from India : Performance , Competitiveness and Determinants. February 2015*.

Rojas-Hernandez, N., Véliz, D., & Vega-Retter, C. (2019). Selection of suitable reference genes for gene expression analysis in gills and liver of fish under field pollution conditions. *Scientific Reports*, *9*(1), 1–8. https://doi.org/10.1038/s41598-019-40196-3

Ronen, A., Perelberg, A., Abramowitz, J., Hutoran, M., Tinman, S., Bejerano, I., Steinitz, M., & Kotler, M. (2003). Efficient vaccine against the virus causing a lethal disease in cultured Cyprinus carpio. *Vaccine*, *21*(32), 4677–4684. https://doi.org/10.1016/S0264-410X(03)00523-1

Rougée, L., Ostrander, G. K., Richmond, R. H., & Lu, Y. (2007). Establishment, characterization, and viral susceptibility of two cell lines derived from goldfish Carassius auratus muscle and swim bladder. *Diseases of Aquatic Organisms*, *77*(2), 127–135. https://doi.org/10.3354/dao01802

Sahoo, P. K., Swaminathan, T. R., Abraham, T. J., Kumar, R., Pattanayak, S., Mohapatra, A., Rath, S. S., Patra, A., Adikesavalu, H., Sood, N., Pradhan, P. K., Das, B. K., Jayasankar, P., & Jena, J. K. (2016). Detection of goldfish haematopoietic necrosis herpes virus (Cyprinid herpesvirus-2) with multi-drug resistant Aeromonas hydrophila infection in goldfish: First evidence of any viral disease outbreak in ornamental freshwater aquaculture farms in India. *Acta Tropica*, *161*, 8–17. https://doi.org/10.1016/j.actatropica.2016.05.004

Saito, H., Okamura, T., Shibata, T., Kato, G., & Sano, M. (2022). Development of a live attenuated vaccine candidate against herpesviral hematopoietic necrosis of goldfish. *Aquaculture*, *552*(February), 737974. https://doi.org/10.1016/j.aquaculture.2022.737974

Sallusto, F., Lanzavecchia, A., Araki, K., & Ahmed, R. (2010). From vaccines to memory and back. *Immunity*, *33*(4), 451–463. https://doi.org/10.1016/j.immuni.2010.10.008

Schoenborn, J. R., & Wilson, C. B. (2007). Regulation of interferon-$γ$ during innate and adaptive immune responses. *Advances in Immunology*, *96*, 41–101.

Shea, T. B. (1983). Enhancement of goldfish virus type 2 replicaton by 1-naphthol, the major hydrolysis product of the pesticide carbaryl. *Applied and Environmental Microbiology*, *46*(5), 1230–1231. https://doi.org/10.1128/aem.46.5.1230-1231.1983

Shea, T. B., & Berry, E. S. (1984). Suppression of interferon synthesis by the pesticide carbaryl as a mechanism for enhancement of goldfish virus-2 replication. *Applied and Environmental Microbiology*, *47*(2), 250–252. https://doi.org/10.1128/aem.47.2.250-252.1984

Shibata, T., Nanjo, A., Saito, M., Yoshii, K., Ito, T., Nakanishi, T., Sakamoto, T., & Sano, M. (2015). In vitro characteristics of cyprinid herpesvirus 2: Effect of kidney extract supplementation on growth. *Diseases of Aquatic Organisms*, *115*(3), 223–232. https://doi.org/10.3354/dao02885

Smith, C., & Khanna, R. (2013). Immune regulation of human herpesviruses and its implications for human

transplantation. *American Journal of Transplantation*, *13*(SUPPL. 3), 9–23. https://doi.org/10.1111/ajt.12005

Stephens, F. J., Raidal, S. R., & Jones, B. (2004). Haematopoietic necrosis in a goldfish (Carassius auratus) associated with an agent morphologically similar to herpesvirus. *Australian Veterinary Journal*, *82*(3), 167–169. https://doi.org/10.1111/j.1751-0813.2004.tb12650.x

Swaminathan, T. R., Raja, A., Dharmaratnam, A., & Nithianantham, S. R. (2021). Comparative sensitivity of three new cell lines developed from gill, liver and brain tissues of goldfish, Carassius auratus (L.) to cyprinid herpesvirus-2 (CyHV-2). *Journal of Virological Methods*, *291*(February). https://doi.org/10.1016/j.jviromet.2021.114069

Thangaraj, R. S., Nithianantham, S. R., Dharmaratnam, A., Kumar, R., Pradhan, P. K., Thangalazhy Gopakumar, S., & Sood, N. (2021). Cyprinid herpesvirus-2 (CyHV-2): a comprehensive review. *Reviews in Aquaculture*, *13*(2), 796–821. https://doi.org/10.1111/raq.12499

Vossen, M. T., Westerhout, E. M., Söderberg-Nauclér, C., & Wiertz, E. J. (2002). Viral immune evasion: a masterpiece of evolution. *Immunogenetics*, *54*(8), 527–542.

Waltzek, T. B., Kurobe, T., Goodwin, A. E., & Hedrick, R. P. (2009). Development of a polymerase chain reaction assay to detect cyprinid herpesvirus 2 in goldfish. *Journal of Aquatic Animal Health*, *21*(1), 60–67. https://doi.org/10.1577/H08-045.1

Wang, F. (2021). *Isolation and characterization of a Cyprinid herpesvirus strain YZ01 from apparently healthy goldfish after rising water temperature*. *2*.

Wang, H., Sun, M., Xu, D., Podok, P., Xie, J., Jiang, Y., & Lu, L. (2018). Rapid visual detection of cyprinid herpesvirus 2 by recombinase polymerase amplification combined with a lateral flow dipstick. *Journal of Fish Diseases*, *41*(8), 1201–1206. https://doi.org/10.1111/jfd.12808

Wang, H., Wen, H., Li, Y., Zhang, K., & Liu, Y. (2018). Evaluation of potential reference genes for quantitative RT-PCR analysis in spotted sea bass (Lateolabrax maculatus) under normal and salinity stress conditions. *PeerJ*, *2018*(9). https://doi.org/10.7717/peerj.5631

Wang, L., He, J., Liang, L., Zheng, X., Jia, P., Shi, X., Lan, W., Xie, J., Liu, H., & Xu, P. (2012). Mass mortality caused by cyprinid herpesvirus 2 (CyHV-2) in prussian carp (carassius gibelio) in China. *Bulletin of the European Association of Fish Pathologists*, *32*(5), 164–173.

Watson, S., Mercier, S., Bye, C., Wilkinson, J., Cunningham, A. L., & Harman, A. N. (2007). Determination of suitable housekeeping genes for normalisation of quantitative real time PCR analysis of cells infected with human immunodeficiency virus and herpes viruses. *Virology Journal*, *4*, 3–7. https://doi.org/10.1186/1743-422X-4-130

Wu, T., Ding, Z., Ren, M., An, L., Xiao, Z., Liu, P., Gu, W., Meng, Q., & Wang, W. (2013). The histo- and ultra-pathological studies on a fatal disease of Prussian carp (Carassius gibelio) in mainland China associated with cyprinid herpesvirus 2 (CyHV-2). *Aquaculture*, *412–413*, 8–13. https://doi.org/10.1016/j.aquaculture.2013.07.004

Xiao, Z., Xue, M., Xu, C., Jiang, N., Luo, X., Li, Y., Fan, Y., Meng, Y., Liu, W., Zeng, L., & others. (2022). First report of cyprinid herpesvirus 2 isolated from the golden crucian carp in China. *Aquaculture*, 738361.

Xu, J., Zeng, L., Zhang, H., Zhou, Y., Ma, J., & Fan, Y. (2013). Cyprinid herpesvirus 2 infection emerged in cultured gibel carp, Carassius auratus gibelio in China. *Veterinary Microbiology*, *166*(1–2), 138–144. https://doi.org/10.1016/j.vetmic.2013.05.025

Xu, Y., Zhou, Y., Wang, F., Ding, C., Cao, J., & Duan, H. (2019). Development of two brain cell lines from goldfish and silver crucian carp and viral susceptibility to Cyprinid herpesivirus-2. *In Vitro Cellular \& Developmental Biology-Animal*, *55*(9), 749–755.

Yan, W., Nie, P., & Lu, Y. (2011). Establishment, characterization and viral susceptibility of a new cell line derived from goldfish, Carassius auratus (L.), tail fin. *Journal of Fish Diseases*, *34*(10), 757–768. https://doi.org/10.1111/j.1365-2761.2011.01292.x

Yan, Y., Huo, X., Ai, T., & Su, J. (2020). β-glucan and anisodamine can enhance the immersion immune efficacy of inactivated cyprinid herpesvirus 2 vaccine in Carassius auratus gibelio: Immersion immune adjuvants. *Fish and Shellfish Immunology*, *98*(January), 285–295. https://doi.org/10.1016/j.fsi.2020.01.025

Yuan, X., Shen, J., Pan, X., Yao, J., Lyu, S., Liu, L., & Zhang, H. (2020). Screening for protective antigens of Cyprinid herpesvirus 2 and construction of DNA vaccines. *Journal of Virological Methods*, *280*(999), 113877. https://doi.org/10.1016/j.jviromet.2020.113877

Zeng, X. T., Chen, Z. Y., Deng, Y. S., Gui, J. F., & Zhang, Q. Y. (2016). Complete genome sequence and architecture of crucian carp Carassius auratus herpesvirus (CaHV). *Archives of Virology*, *161*(12), 3577–3581. https://doi.org/10.1007/s00705-016-3037-y

Zhang, H., Zeng, L., Fan, Y., Zhou, Y., Xu, J., & Ma, J. (2014). A loop-mediated isothermal amplification assay for rapid detection of cyprinid herpesvirus 2 in gibel carp (Carassius auratus gibelio). In *The Scientific World Journal* (Vol. 2014). https://doi.org/10.1155/2014/716413

Zhang, L., Ma, J., Fan, Y., Zhou, Y., Xu, J., Liu, W., Gu, Z., & Zeng, L. (2016). Immune response and protection in gibel carp, Carassius gibelio, after vaccination with β-propiolactone inactivated cyprinid herpesvirus 2. *Fish and Shellfish Immunology*, *49*, 344–350. https://doi.org/10.1016/j.fsi.2016.01.003

Zhang, Y., Zhang, X., Liang, Z., Zhu, M., Zhang, M., Zhang, T., Zhang, Z., Cao, M., Gu, Y., Pan, J., Yan, B., Zhu, H., Xue, R., Cao, G., Gao, Y., Hu, X., & Gong, C. (2020). Expression pattern and regulatory network of gibel carp (Carassius gibelio) miRNAs and their putative target genes in response to CyHV-2 infection. *Aquaculture*, *523*(February), 735178. https://doi.org/10.1016/j.aquaculture.2020.735178

Zhou, Y., Jiang, N., Ma, J., Fan, Y., Zhang, L., Xu, J., & Zeng, L. (2015). Protective immunity in gibel carp, Carassius gibelio of the truncated proteins of cyprinid herpesvirus 2 expressed in Pichia pastoris. *Fish and Shellfish Immunology*, *47*(2), 1024–1031. https://doi.org/10.1016/j.fsi.2015.11.012

Zhu, M., Li, K., Xuan, Y., Sun, Z., Liu, B., Kumar, D., Jiang, M., Pan, Y., Zhang, Y., Gong, Y., Lu, X., Yu, D.,

Hu, X., Cao, G., Xue, R., & Gong, C. (2019). Host range and vertical transmission of cyprinid herpesvirus 2. *Turkish Journal of Fisheries and Aquatic Sciences, 19*(8), 645–652. https://doi.org/10.4194/1303-2712-v19_8_02

Zhu, M., Liu, B., Cao, G., Hu, X., Wei, Y., Yi, J., Zhou, Y., Pan, G., Wang, J., Xue, R., & Gong, C. (2015). Identification and rapid diagnosis of the pathogen responsible for haemorrhagic disease of the gill of Allogynogenetic crucian carp. *Journal of Virological Methods, 219*, 67–74. https://doi.org/10.1016/j.jviromet.2015.03.019

Ziegler, S. F. (2016). Division of labour by CD4+ T helper cells. *Nature Reviews Immunology*, *16*(7), 403.

List of publications from thesis
List of conferences presented (from thesis)

S. No	Authors	Title of Research Publication	Title of Journal	Year of Publications
1	**Arathi Dharmaratnam**, Arun Sudhagar, T.Raja Swaminathan	Evaluation of protective effects of heat-inactivated cyprinid herpesvirus-2 (CyHV-2) vaccine against herpesviral hematopoietic necrosis disease (HVHND) in goldfish (Carassius auratus)	Fish and Shellfish Immunology	2022
2	**Arathi Dharmaratnam** , Arun Sudhagar, N. Sundar Raj, Sweta Das, T.Raja Swaminathan	Evaluation of candidate reference genes for quantitative RTqPCR analysis in goldfish (Carassius auratus L.) in healthy and CyHV-2 infected fish.	Veterinary Immunology and Immunopathology	2021
3	**Arathi Dharmaratnam** , Raj Kumar , Basheer Saidmuhammed Valaparambil , Neeraj Sood , Pravata Kumar Pradhan, Sweta Das and T. Raja Swaminathan	Establishment and characterisation of fantail goldfish fin (FtGF) cell line from goldfish, Carassius auratus for in vitro propagation of Cyprinid herpes virus-2 (CyHV-2). Peer J 8: e937	Peer J	2020
4	**Arathi Dharmaratnam** ,Arun Sudhagar, Reshma r Nair, N. Sundar Raj, Preena P. G, T.Raja Swaminathan.	Protective efficacy of formalin inactivated whole cell of CyHV-2 vaccines and their immune gene response.	Microbial pathogenesis Communicated Status - Revision	
5	T.Raja Swaminathan, N. Sundar Raj, **Arathi Dharmaratnam**, Raj Kumar, Pradhan Pravatha Kumar, T.G. Sumithra, Neeraj Sood	Cyprinid Herpes virus-2 (CyHV-2) : a comprehensive review	Reviews in aquaculture	2020
6	Sweta Das, **Arathi Dharmaratnam,** Charan R, Kumar R, T.Raja Swaminathan	Immune genes expression in Cyprinid Herpesvirus-2 (CyHV-2) sensitized peripheral blood leukocytes (PBLs) co-cultured with CyHV-2-infected goldfish fin cell line	**Aquaculture International**	2021
7	T.Raja Swaminathan, Arun Raja, **Arathi Dharmaratnam** N. Sundar Raj	Comparative sensitivity of three new cell lines developed from gill, liver and brain tissues of goldfish, *Carassius*	Journal of virological methods	2021

		auratus (L.) to cyprinid herpesvirus-2 (CyHV-2)		
8	T.Raja Swaminathan, Preena P. G, Arun K, Tina, **Arathi Dharmaratnam**	Quick hassle-free detection of cyprinid herpesvirus 2 (CyHV-2) in goldfish using Recombinase Polymerase Amplification - Lateral Flow Dipstick (RPA-LFD) assay.	Aquaculture International	2021

S.No	Conference period	Name of coference	Title of paper	List of Authors	Oral/ Poster
1	2021(20-24 Sep)	World Fisheries Congress	Immune gene response and protective efficacy of formalin inactivated whole cell of CyHV-2 vaccines.	**Arathi Dharmaratnam**, Arun Sudhagar, N. Sundar Raj, Preena P. G, T.Raja Swaminathan.	Oral
2	2020 (11-14 Feb)	CLIMFISHCON organized by CUSAT	Evaluation of candidate reference genes for quantitativeexpression studies in goldfish (*Carassius auratus l.)*.	**Arathi Dharmaratnam**, Preena P.G, Sundar Raj N, Raja Swaminathan T. Kuldeep K. Lal.	Oral
3	2019 (16-20 Dec)	MARICON organized by CUSAT	Comparison of FtGF, FtGG, FtGB and FtGK cell line derived from different tissues of goldfish in the propagation of cyprinid herpes virus -2	**Arathi Dharmaratnam,** Arun Raja S, Sundar Raj N and Raja Swaminathan T.	Oral
4	2017(21-24 Nov)	11th Indian Fisheries and Aquaculture Forum., Kochi organized by ICAR-CIFT, Kochi	Establishment of highly permissive cell line GFF for continuous propagation of CyHV-2 virus	**D.Arathi**, Raj Kumar, Charan Ravi, V S Basheer and Raja Swaminathan T	Oral
5	2017(20-21 Apr)	ISAAHE organized by NBFGR, Lucknow	Development of highly specific and sensitive nested PCR primers for detection of herpesviral hematopoietic necrosis virus in goldfish , Carassius aurutus	**Arathi Dharmaratnam**, Raja Swaminathan T, Raj Kumar, Charan Ravi, Basheer V. S, Pradhan P, Neeraj Sood.	Poster
6	2016(8-10 Dec)	Virocon organized by ICAR- IIHR, Bangalore	Cyprinid Herpesvirus 2(CyHV 2) in India: .Presence of new strain.	**Arathi Dharmaratnam**, Raja Swaminathan T, Raj Kumar, V S Basheer, P K Pradhan and Neeraj Sood. 2016. Cyprinid Herpesvirus 2(CyHV 2) in India.Presence of new strain	Poster